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ISOPROPYL ALCOHOL IN THE PARAFFIN INFILTRATION TECHNIC

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ABSTRACT.—**A method is described for using isopropyl alcohol for dehydration of animal tissues preceding melted paraffin infiltration. Advantages of the technic are: simplicity, low cost, low toxicity, and diminished distortion and hardening of the tissues.**

Isopropyl alcohol has been used for many years in histological technic as a substitute for ethyl alcohol to dehydrate tissues; see Griffin (1922), Bradbury (1931), Herman (1941). These authors all reported that isopropyl alcohol is either equal or superior to ethyl alcohol in performance. Reasons motivating the substitution are that isopropyl alcohol is readily available at low cost, and has been found to shrink and harden animal tissues less. Isopropyl alcohol does not have the undesirable toxic effects of dioxane; as it is unpalatable, it is free of internal revenue restrictions.

In 1929, Sheridan (1929) published his technic of using normal propyl alcohol and low melting point paraffin in paraffin infiltration. The method of using isopropyl alcohol, herein described, is quite similar. The substitution of isopropyl alcohol for normal propyl alcohol is made because of its low cost and ease of acquisition.

The published technics have not included the direct transfer of tissues from isopropyl alcohol to melted paraffin, but used an intermediate paraffin solvent or clearing agent. This intermediate step, which may cause hardening and distortion, is unnecessary. While cold paraffin is nearly insoluble in isopropyl alcohol, melted paraffin is completely miscible. Because of the low boiling point and low specific gravity of isopropyl alcohol, it is readily and completely expelled from both the tissues and the paraffin baths.

The schedule given below is one particularly adapted to the

author's working hours. For the working conditions in other laboratories a modification of the schedule might be advisable. Formalin-fixed animal tissues are washed overnight to remove the fixative. They are dehydrated, infiltrated and embedded as follows:

(1) Isopropyl alcohol, 60%..... 1 hr.
(2) Isopropyl alcohol, 99%..... 1 hr.
(3) Isopropyl alcohol, 99%..... 2 hr.
(4) Isopropyl alcohol, 99% (40–45°C.)..... 3 hr.
(5) Melted Tissuemat (M.P. 50–52°C.)..... 1 hr.
(6) Melted Tissuemat (M.P. 50–52°C.)..... Overnight
(7) Embed and block in tissuemat of right melting point to suit cutting conditions.

After using this embedding technic, tissue sections 8μ thick are easily prepared and differential staining is not impaired.

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DIFFERENTIAL IN TOTO STAINING OF BONE, CARTILAGE AND SOFT TISSUES

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ABSTRACT.—**The following method for staining bone and cartilage allows study of the gross cleared specimen and does not injure the tissues for subsequent microscopic study: Fix in 10% neutral formalin; bleach thoroughly in 3% H_2O_2 in sunlight. Wash in distilled water. Stain bone 24 hours in 0.01 g. of Biebrich scarlet in 100 ml. of distilled water. Destain in 95% alcohol until soft tissues and cartilage are colorless. Stain cartilage 24 hours in a pH2 buffer solution of 2.1g. of citric acid per 100 ml. of water with 0.001 g. of methylene blue. Destain in pH2 buffer solution until soft tissues are pale. Dehydrate in two changes of 95% alcohol in preparation for clearing. (This completes the destaining and may remove too much stain from the cartilage if destaining in the pH2 solution has been carried too far.) Place in Groat's clearing fluid and cover loosely so that the alcohol may evaporate, or remove the alcohol in vacuo. Groat's Mixture No. 19 is usually satisfactory.**

For a combined stain, first stain bone, as above, and then apply the cartilage stain.

Seal jars with an ordinary liquid wood glue such as LePage's.

INTRODUCTION

In a study of fetal skeletal deformities which the author was undertaking, it soon became apparent that it would be advantageous first to study the specimens grossly with the bone and cartilage stained and the soft tissues transparent, and then to follow this with microscopic examination of the same specimens. This paper presents methods of selective *in toto* staining of bone and cartilage, which when combined with Groat's (1941) method of clearing, do not damage the tissue for subsequent microscopic study.

REVIEW OF THE LITERATURE

Clearing methods: Schultze's method of clearing as described by Hill, (1906) depends upon maceration of the soft tissues in 1% KOH and has the disadvantage of making subsequent microscopic study impossible.

[1]This work was done in the Department of Anatomy of the University of Wisconsin while the author was an Orthopedic Resident in the Department of Surgery

Several clearing methods are based upon the principle of immersing the specimen in a liquid with a refractive index similar to that of the soft tissue of the specimen. Spalteholtz (1914) recommended methyl salycilate and Lundvall (1904) used carbon disulfide. Groat (1941) achieved maximum clearing by adjusting the refractive index of a mixture of triorthocresyl phosphate and tributyl phosphate to that of the tissue.

Bone stains: Schultze's alizarin bone stain, as described by Hill (1906), depends upon maceration of the soft tissues with KOH until they no longer take the stain. Batson (1921) found a number of dyes suitable for Schultze's method. Williams (1941) combined a cartilage stain with Schultze's bone stain.

Cartilage stains: Lundvall (1904) described a cartilage stain with an acidified alcoholic solution of toluidine blue. He credited the stain to Van Wijhe. Da Rocha (1917) described additional dyes for this stain. Miller (1921) used the KOH method of clearing with this method. Hamberger (1940) described a similar stain using methylene blue.

Experimental Work

Buffer solutions from pH2 to 11 at intervals of 1 pH unit were made up by the methods of McIlvaine and of Kolthoff and Vlesschhower as given by Clark (1928). Dyes were tested in the buffer solutions and in distilled water. Alcohol, acetone and acid alcohol were tried in destaining. Test objects were split vertebrae of 10 cm. fetal pigs fixed in neutral formalin and bleached in H_2O_2.

Bone stains: A group of dyes classed as "acid" by Conn (1940) stained bone differentially only in distilled water (pH6). Destaining was done in 60% to 95% ethyl alcohol. There was one exception: fast green FCF stained bone not only at pH of distilled water (pH6) but throughout the whole range to pH11. Since this dye is an indicator the stain was green at pH6 and blue at pH11. Because all bone stains were slightly transparent they were less striking than Schultze's alizarin stain.

Cartilage stains: A group of dyes classed as "basic" by Conn (1940) stained only cartilage at pH2. Destaining was best in citric acid in water at pH2, or in 60% to 95% alcohol. In distilled water a fast stain of all tissues occurred. There was no stain at pH11. Cartilage could be successfully stained following the bone stain, but if the cartilage stain was performed first the bone stain produced a fast stain of all tissues, as though it had taken place at pH2. Attempts to wash out or neutralize the acid used in the cartilage stain were unsuccessful.

Staining Method

Preparation of specimens: Always remove the liver since it is almost impossible to bleach and its pigment darkens the clearing fluid. The staining and destaining process depends upon diffusion of solutions through the tissues and is facilitated by complete evisceration and by opening the cranial cavity through the fontanelles. A sagittally split specimen is excellent and demonstrates the skeleton without overlap. In large specimens incisions into large muscle masses such as occur in the neck facilitate diffusion.

Fixation and bleaching: Fix well in 10% formalin made neutral by addition of an excess of calcium carbonate. The presence of traces of formic acid interferes with the bone stain. Bleach thoroughly in several changes of 3% H_2O_2 in distilled water in sunlight. This may take several weeks. Wash 24 hours in distilled water. Bubbles in the tissue may be removed in vacuo or with a needle and syringe.

Concentration of dye used: The optimum dye concentrations are given in Table 1. Deviations from the optimum concentration of about 5× may produce a pale stain or make destaining difficult; e.g. orange II 0.01%—pale; 0.05%—sharp; 0.1%—soft tissue difficult to destain. In general more mature specimens require a more dilute bone stain and a more concentrated cartilage stain.

General: The timing and concentrations of dyes listed below are for an eviscerated 10 cm. pig fetus.

Repeated staining and destaining destroy the affinity of the tissues for the dye.

Bone stain: Stain 24 hours in a solution of any one of the dyes made up according to Table 1. Biebrich scarlet is very good.

Destain in alcohol (60% to 95% is suitable) until soft tissues and cartilage are colorless. The color shining through from underlying bone, particularly over the long bones, may lead to excessive destaining and should be disregarded.

Cartilage stains: Stain 24 hours in a solution of any one of the dyes made up according to Table 1. Methylene blue is very good.

Destain in pH2 buffer solution (21 g. citric acid per 1 liter distilled water) until soft tissue is pale. The final dehydration with 95% alcohol in preparation for clearing will complete the destaining and may remove too much stain from the cartilage if destaining in the pH2 is overdone.

Combined stain of bone and cartilage: Use the bone stain first, and, when destaining is complete, wash in a few changes of distilled water until the specimen sinks. Then stain for cartilage and clear.

Clearing: After staining dehydrate the specimen in two changes of 95% alcohol. Place in Groat's clearing fluid and cover loosely so that the alcohol may evaporate, or remove the alcohol in vacuo. Groat's mixture No. 19 is usually satisfactory.

Fluorescent dyes: Fluorescent dyes aid in the study of surface contours, since visible fluorescence occurs almost entirely at the surface of the fluorescing object or solution. Direct or oblique illumination

TABLE 1.—THE AMOUNT OF DYE AND CITRIC ACID TO USE PER LITER OF DISTILLED WATER IN MAKING UP THE BONE AND CARTILAGE STAINING SOLUTIONS.
Some of the characteristics of the stains are also given.

DYES	G. per liter	Citric acid G. per liter	Color of stained tissue	Relatively fast stains*	Fluoresces under "glass" ultra violet	Soft tissues difficult to destain
BONE STAINS						
Benzopurpurin (G)	0.1	—	Red			
Biebrich scarlet (NA)	0.1	—	Red	+		
Bordeau red (G)	0.1	—	Red	+		
Eosin Y (NA)	0.1	—	Orange		+	
Fast green FCF (C & B)	0.1	—	Green†	+	+	
Orange II (NA) (Cert. NOb-2)	0.5	—	Yellow	+		
Ponceau red (NA)	0.1	—	Red	+		
CARTILAGE STAINS						
Cresyl echt violet (NA)	0.02	2	Blue		+	+
Gentian violet (G)	0.02	2	Blue			+
Janus green (NA) (Cert. NJ-1)	0.02	2	Blue			
Methylene blue (NA)	0.01	2	Blue			
Nile blue (NA)	0.02	2	Blue	+		+
Thionin (G)	0.02	2	Blue			+
Toluidin blue (G)	0.02	2	Blue			
Victoria blue (G)	0.02	2	Blue			+

*Specimens stained by those indicated as fast had not faded appreciably after three months of exposure in a sunny window. The others fade slowly if exposed to light. With a glass electrode the bone stain solutions prove to be pH 6.0±0.2, and the cartilage stain solutions pH 2±0.1.

†Fast green will produce a blue stain if made up at pH 11 by buffering with 5.3 g. of Na_2CO_3 per liter. Sources of dyes are indicated as follows: (C & B) Coleman and Bell; (G) Gruebler; (NA) National Aniline & Chemical Co.

must of course be used instead of transmitted light. Suitable sources of near or "glass" ultra-violet are sunlight, or any white burning or overloaded electric light, such as a focusing microscope lamp or a flashlight with new batteries. True ultra-violet is unsuitable since it causes all tissue to fluoresce white, even though it is cleared and not stained.

Fluorescent bone stain: A brilliant fluorescent bone stain is produced by staining 24 hours in a saturated alcoholic solution of fluorescein

(Gruebler) and destaining with alcohol. This is unsuitable for combined stains as it destains during cartilage staining. The stain also fades rapidly if exposed to sunlight.

Fluorescent soft tissue stain: Stain with eosin Y, 1 g. and citric acid, 21 g. per liter H_2O (distilled). Destaining is not necessary. Surface fluorescence in cleared specimens from tendons, blood vessels and nerves outlines their course when examined by reflected light.

Demonstration of epiphyseal lines by the cartilage stains: In specimens from adolescents only the epiphyseal lines and a thin layer of cartilage surrounding the epiphysis stain. The growing cap of cartilage on osteochondromas stains similarly. In fetal specimens decalcification with 1% HNO_3 reduces the affinity of cartilage for the dye except at the epiphyseal plate. Prolonged HNO_3 treatment destroys the staining quality of all cartilage. This varies greatly with the maturity of the specimen, so no definite treatment time can be specified. Adult articular cartilage does not stain by this method.

Unsatisfactory stains: Stains tried and found to be unsatisfactory for these methods are the following: Acid fuschin, acid green, acriflavine, alizarin red S, amaranth, anilin blue W.S., aurantia, azure A, basic fuchsin (magenta red), Bismarck brown, carmine, chrysoidin (Grübler), dianil blue, "erythrosin pur" (Grübler), fast acid blue, hematein, iodine green, malachite green, mercurochrome, methyl green, methyl red, naphthaline blue, nigrosin, nitrazine, orange G, safranin, Sudan IV (scarlet red), Trypan blue.

Sealing specimen jars: Since Groat's clearing mixture is not volatile, it is not essential to seal jars. Ordinary LePage's wood glue is the best sealing agent for this solution we have found. The surfaces to which the glue is applied must be cleaned and dried with acetone or some similar solvent of the clearer, and the clearing agent must be kept from contact with the glue until it has dried. The usual sealing compounds are dissolved by the clearing solution.

Sectioning subsequent to in toto clearing: Tissues from stained specimens cleared by Groat's method may be infiltrated with paraffin directly from the triorthocresyl and tributyl phosphate mixture. Ordinarily it would probably be more satisfactory to wash out the clearing agent with some more volatile substance such as dioxane or cyclohexanone before placing the tissue in paraffin. Sections must be restained, as the processing removes most, if not all, of the *in toto* stain.

Discussion

This staining method is presented on a purely empirical basis. Time did not permit further exploration of the problem or application

of the method to microscopic sections. The work of Silver (1942) with silver stains indicates that the isoelectric point of the various proteins is the determining factor in selective staining. Under high dry magnification the cartilage stain appears to be in the matrix, since rows of clear spaces corresponding to the cartilage cell columns were seen. There was a clear area 20 to 30 μ wide between the cartilage and the stained bone. Whether the bone stain depends upon the presence of calcium could not be determined since decalcification changed the specimen so that bone, cartilage and soft tissue stained.

Summary

A method of *in toto* staining of fetal bones and cartilage which allows subsequent microscopic study of the specimen is presented. Control of pH is essential to the success of the method. Fluorescent stains for bone, cartilage and soft tissue are given, and also a method of staining epiphyseal plate cartilage. Groat's method of clearing is recommended.

Acknowledgement

The author wishes to thank Dr. H. W. Mossman for suggesting this problem and for his constant help during its development.

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A UTILIZATION OF THE KOHLER SYSTEM OF ILLUMINATION

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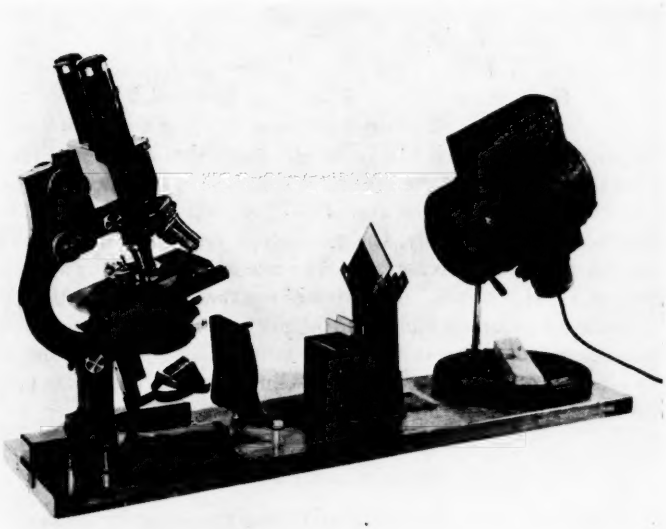
The following system of microscope lighting is based on ideas gleaned from a number of sources, particularly Belling (1930) and Shillaber (1944). The principles are not new and this article is primarily concerned with an implementation of the well known Kohler method. The chief points to be emphasized are the use of a prism and a polaroid variable density screen.

In lieu of an optical bench the components are mounted on a board. The light source is a six volt ribbon filament lamp in a housing equip-



ped with condensing lenses and an adjustable diaphragm. If a silvered mirror or other reflector is located in the lamp, it should be removed so that the only effective illumination is directly from the ribbon filament. The lamp is located about fifteen inches from the substage mirror.

In the middle of the board is a filter-holding rack made of small pieces of angle iron inserted in slots cut into the support. Next to it is a filter-holding box, built to hold filters made of 2×2 inch Wrat-

ten gelatin cemented between 2×3 inch microslides. The lips of the compartments are lined with velvet removed from discarded film packs. The filters are thus dusted and protected from light when inserted in the box. Wratten filters 61, 57A and 66 are used by the writer.

Immediately in front of the substage mirror is mounted a polaroid variable density screen. Any device by which one polaroid element can be rotated in reference to another will serve the purpose. The writer used a variable density screen removed from an Army Air Force gunsight[1] that ordinarily is mounted permanently in the line of vision of pursuit pilots by means of four bolts. Disassembly of the gunsight and judicious use of a hack saw will remove the variable screen with its hinges and two of the bolt mountings. Thus, the screen may be swung out of the optical axis to obtain maximum lighting.

A first surface mirror or a prism is very desirable in removing the secondary light images formed by the first surface of the ordinary mirror. Here, an air surfaced 90° prism is mounted, by use of Clarite, on the plane mirror. The silver of the mirror now serves as a part of the prism, converting it to a "silvered" prism that requires no further mounting. The entrance surface of a silvered prism need not be at perfect right angles to the axis of the light source to give good light reflection. Consequently, the microscope may be tilted as desired without marked disadvantage. The size of the reflecting surface of the prism is 34×23 mm. If a prism of less than 34 mm. length is used, it should be mounted higher on the mirror, since the optical axis of most microscopes meets the tilted mirror above its swinging axis. An alternative would be to follow Belling's suggestion (1930, p. 138) and shorten the stem of the mirror fork.

Operational Procedure

(1) Open diaphragm of lamp and focus the image of the incandescent ribbon filament on the diaphragm of the substage condenser. This need not be repeated except for occasional checking. Close diaphragm to about one half inch diameter.

(2) Bring material on slide into focus.

(3) Adjust the substage condenser until edges of the lamp diaphragm are in focus. Open the lamp diaphragm to just beyond the visual field. (N. B. If it is desired to illuminate the entire field of a 16 mm. objective by the Kohler system, the lamp will usually need to

[1]Obtained from Edmund Salvage Co., Audubon, N. J. (Cat. No. 908–W).

be less than 15 inches from the mirror. The writer switches to diffuse lighting by placing a frosted glass in the filter holder.)

(4) Adjust the condenser diaphragm to best *resolution*, avoiding glare in one extreme and diffraction bands in the other.

(5) Adjust the polaroid elements to obtain a satisfactory *intensity* of light.

(6) Insert filters for desired contrast and readjust polaroid settings.

In principle the condenser diaphragm should be used only to adjust the cone of light from the condenser to the numerical aperture of the particular objective in use. The *intensity* of light is then regulated by other means; by neutral filters, or, in this case, by polaroid. The latter point is of importance in photomicrography. Changes can be made in the amount of transmitted light without altering the quality of the image.

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AN ADAPTABLE STAINING SCHEDULE FOR PLANT TISSUES

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Abstract.—**A general schedule for staining meristematic, maturing, and mature plant tissues is described. Treatment with a dilute aqueous solution of Delafield's hematoxylin is followed with staining in 0.1% safranin in 60% alcohol. Destaining of safranin may be partly accomplished in alcohol and completed by counterstaining with dilute fast green FCF in a xylene and alcohol mixture. Various modifications and adaptations are briefly discussed.**

Preparation of good-quality research or teaching slides from paraffin-embedded tissues requires tedious and time-consuming manipulation when the usual staining methods are employed. In the course of several years various modifications of previously reported methods have been used and combined. The result has been the development of a schedule which is fairly rapid without sacrifice of permanency and sharp differentiation, and by which destaining problems are reduced to a minimum. In addition, the schedule has proved to be quite adaptable and has produced good results with a wide range of plant tissues. Differentiation of cell contents, nuclei, and cell walls is sharply produced in apical meristems, embryos, and other tissues which have been killed and fixed, at various stages of development, in formalin-acetic-alcohol and in Karpechenko's solutions (Rawlins, 1933).

After removal of paraffin from the sections with xylene, the slides are placed in absolute alcohol for a few seconds. They are then removed, the backs are wiped dry, and the alcohol is allowed to evaporate from the sections. Better results can be secured if, as the alcohol has evaporated, the same procedure is repeated with fresh alcohol. Contrary to the usual notion that once the paraffin has been removed the sections must not be allowed to dry, this procedure appears to be safe for plant tissues. Virtually complete dehydration of the tissues has already occurred during prior stages of preparation. Also, there is the advantage, in allowing the sections to become dry at this stage, that no trace of xylene is carried through the remainder of the series. The slides are then transferred to a Coplin jar of 95% ethyl alcohol and are ready for the following general schedule:

[1]Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

(1) Water rinse. (May include a mordant).

(2) Delafield's hematoxylin (stock solution diluted to about 10% with distilled water), 5 to 15 minutes according to requirements. Avoid overstaining.

(3) Water rinse (or differentiating agent followed by water rinse).

(4) Safranin O, 0.1%, in 60% ethyl alcohol (addition of a few drops of 5% aqueous solution of sodium acetate will greatly improve the staining quality of the safranin solution). This dilution of safranin considerably minimizes the problems of overstaining and destaining. Some tissues are sufficiently stained in half an hour; others require at least two hours. The procedure may be hastened by use of warm solution in a paraffin oven for 5 to 15 minutes. Or, if it is more convenient to do so, sections may be left overnight or longer in the solution (at room temperature) without becoming too heavily stained.

(5) Water rinse.

(6) Destain in alcohol. (If necessary, a drop or two of 1 or 2% hydrochloric acid may be added to the alcohol. Destaining is slower in 95% alcohol than in more dilute solutions.)

(7) Fast green serves as a destaining agent as well as a counterstain, and is easily controlled in the described solution. A few ml. of a saturated solution of fast green FCF in clove oil and alcohol (1:1) are added to a Coplin jar containing 50 ml. xylene and alcohol in a ratio of about 3:1. Generally, 1 to 3 minutes is sufficient to complete the destaining of the safranin to a desired stage and also to provide a good counterstain. The safranin staining may be adjusted according to results obtained with fast green.

(8) Xylene-alcohol rinse (two different Coplin jars).

(9) Two changes in xylene before mounting in balsam or a suitable synthetic resin.

This schedule may be modified by the use of Foster's (1934) technic with tannic acid and ferric chloride, in place of Delafield's. Some objection may be found to the results of the tannic acid and ferric chloride method, in that the ferric deposits in the middle lamellae and adjacent wall regions may be too heavy; thus the finer details, which are well differentiated with Delafield's technic, may be obscured. A part of this difficulty can be overcome with tannic acid and ferric chloride in solutions of 0.1%. About one minute in each solution generally is sufficient to define sharply the walls of meristematic cells, but the sections should be thoroughly rinsed after immersion in tannic acid solution and before they are placed in ferric chloride.

Delafield's formula also may be used following the tannic acid and ferric chloride treatment which serves additionally as a mordant. Several other hematoxylin preparations and mordants described by Cole (1943) also have been used in this schedule with excellent results. In fact, hematoxylin preparations, including alcoholic as well as aqueous solutions, can be manipulated to produce black nuclei, bluish

cytoplasm, and lavender cellulose walls; or (when combined with safranin and fast green) black or pink nuclei with deep red nucleoli, green or blue cytoplasm, lavender, or black cellulose walls, and red lignified walls can be obtained.

Northen (1936) has described several modifications of Foster's method which in part are also adaptable to the above schedule. Sharman (1943) has described a schedule using very dilute safranin in combination with orange G, tannic acid, and iron alum. Boke (1939) and Cross (1937) previously have reported methods using Delafield's preparation and fast green in a manner similar to those described here. Boke used safranin in xylene and alcohol with good results. The use of warm safranin for plant tissues has been previously reported by Shapiro (1947).

The combination of both hematoxylin and fast green with dilute safranin, as described here, provides a schedule of greater adaptability than do most methods. Structures containing closely associated meristematic, maturing and mature cells may be stained with uniformly good results for the different sorts of tissue present. In addition, the more delicate structures are well defined without loss of detail and may be easily photomicrographed under proper conditions of illumination.

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A NEW STAIN MIXTURE: ACETO-ORCEIN-FAST-GREEN

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Abstract.—**A new mixture consisting of aceto-orcein, fast green, and salt is described. The mixture provides contrast between nuclear material, nucleoli and cytoplasm without differentiation. We have found it most useful in the staining of teased tissues, squashes, smears and suspensions (fixed and unfixed). We recommend it for the great speed and simplicity with which it provides a stain having the contrast of much more laborious histological technics.**

Aceto-orcein (La Cour, 1941) has for some time been a favorite stain of the cytologist. Its simplicity and speed, especially for fresh material, have made for its great popularity. However, when counterstaining is desired, it has been the practice to make a permanent preparation of the aceto-orcein stained preparation before staining with fast green. In an effort to preserve the advantages of speed and simplicity, we have developed a dye mixture for contrast staining.

The mixture is prepared from materials usually on hand in the laboratory:

1% orcein in 45% acetic acid[2]	27 ml.
1% fast green in alcohol	3 ml.
2*M* NaCl	2 ml.

The greenish-purple mixture is applied to the preparation for a few minutes, covered with a cover slip, and examined (excess stain may be removed by light pressure over the cover slip with filter paper). Chromatin stains brownish-red, nucleoli and cytoplasm green. Thus, a preparation comparable to that obtained with the Feulgen stain counterstained with fast green is obtained in a few minutes, and with only one staining operation. The method is particularly applicable to teased tissues which are not readily fixed to the slide.

The addition of salt is essential for uniform staining. It was found, from experiments with fast green, that fast green stained much more intensely in the complete absence of salt than in its presence (Kurnick, 1947a). As a result, if material suspended in a salt-free medium

[1]This work was done in part under an American Cancer Society fellowship (N. B. Kurnick) recommended by the Committee on Growth of the National Research Council.

[2]Two samples of orcein were studied: a) Grübler and (b) Coleman and Bell, crystallin, C. P. The latter sample, perhaps because of its less ready solubility, stains somewhat less intensely and the stained chromatin is of a browner shade than is obtained with the Grübler stain. Otherwise entirely similar results were obtained... The Fast Green FCF used was manufactured by National Aniline, Cert. No. NGf8.

(as acetic-alcohol) is stained with the aceto-orcein-fast-green mixture from which *salt* has been *omitted*, the characteristic differential staining is obscured by over-all dense staining with the fast green. The addition of salt to the stain itself has obviated this difficulty.

We have applied this stain mixture to fresh teased onion root tips and to onion root tips preserved in acetic alcohol. They stain alike with contrast as described above: brownish-red chromatin, green nucleoli and cytoplasm. Fresh and fixed grasshopper testes likewise stain similarly. Here again, a remarkable similarity to the Feulgen stain (counterstained with fast green) is noted in that immature sperm cells stain with the usual contrast between brownish-red chromatin and green nucleoli and cytoplasm, and the mature spermatazoa appear as reddish filamentous heads with pale green tails. Connective tissue stains pale green. Nuclei isolated from beef thymus and liver by the citric acid technic (Stoneburg, 1936) and suspended in citric acid, saline, or acetic-alcohol all demonstrated the expected staining characteristics with the mixture. We have found it very useful in checking on the purity of isolated chromosomes (Mirsky and Ris, 1947) since the chromatin threads reveal no green staining, while nucleoli, contaminating cytoplasmic and connective tissue debris stain green. Paraffin sections of mammalian organs have been passed through xylene, and alcohol to water, covered with the stain mixture for five minutes and then examined. Again the characteristic differentiation between chromatin and other structures is obtained. The chromatin stains brownish red; cytoplasm, nucleoli, collagen, and erythrocytes, green. Satisfactory permanent preparations were obtained by removing the cover glass and dipping the slide successively in 70% alcohol, 95% alcohol, 100% alcohol, xylene and mounting in Clarite. The alcohol removes some of the orcein, but enough is retained by the chromatin to provide a satisfactory stain while the green of the other structures stands out even more brilliantly (with the overlying layer of stain washed away).

Thus, the aceto-orcein-fast-green-salt mixture provides a simple stain mixture which gives contrast between cellular structures without washing or differentiation. The stain should find its greatest use in fresh teased material, squashes, smears and suspensions of tissues in fluid.

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A RAPID STAIN METHOD FOR DETECTING CERTAIN PLANT VIRUS INCLUSIONS

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ABSTRACT.—**The trypan blue method for detecting certain virus crystallin inclusions is greatly facilitated by a pre-stain treatment with a diethyl-ether–ethyl-alcohol solution made up in physiological saline (5 ml. of diethyl ether and 10 ml. of ethyl alcohol diluted to 100 ml. with physiological saline solution). Maceration of the study material in trypan blue solution also gives good results.**

INTRODUCTION

The detection of the presence of certain virus inclusions in host plants became a relatively simple laboratory procedure when McWhorter (1941 a & b) discovered that Phaseolus virus 2 and various lily viruses produced crystallin inclusions which could be stained with an aqueous solution of trypan blue. Subsequent studies of the inclusions of Phaseolus virus 2 resulted in the perfection of more rapid modifications of the original technic.

TECHNICS

The tissue studied, in the majority of cases, was integumentary, consisting of epidermal peelings from diseased and comparable healthy leaves. When these strippings were placed in 0.05% trypan blue in physiological saline solution (1941b), with no pre-stain treatment, 30 minutes to 3 hours were required for the staining reaction to reach its end-point, i.e., a brilliant blue coloration of nuclei. The length of staining time seemed to correlate directly with the age of the plant tissue. Trials showed that crushed cells stained readily, indicating that the foliar cells became less permeable to trypan blue as the plants aged. The problem of increasing permeability was solved by a two-minute pre-stain treatment of the tissue with an aqueous solution of 5% diethyl ether and 10% ethyl alcohol. This solution is made as follows: To 50 ml. physiological saline solution add 10 ml. of 95% ethyl alcohol. To this mixture add 5 ml. of diethyl ether and stir until solution is complete. Make up to 100 ml. with physiological saline solution. If excessive plasmolysis occurs, decrease ether content to 3% and increase alcohol content to 15%.

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The use of this solution resulted in a standard, dependable technic which reached a stain reaction end-point within 3 to 4 minutes after obtaining the leaf strippings. The two-minute treatment in ether-alcohol solution caused very little plasmolysis. Staining in trypan blue without previous fixation resulted in varying degrees of nuclear swelling.

Another method, referred to as the *crush* technic, was used for plants from which it was difficult to obtain leaf strippings. A small piece of leaf or stem is placed in a drop or two of 0.05% trypan blue on a slide and then rubbed until macerated with a specially prepared glass spatula having a flat ground glass surface. The preparation is then ready for immediate observation since the remaining stain serves as a corrective white light filter. The nuclei swell, but the size and shape of the crystallin inclusions are unaffected. Very often the nuclei may be observed floating in the mounting medium with clearly visible, well-stained crystals contained in their nucleoli. Less often, viroplasts have been observed floating free or attached to nuclei.

The drastic crush method is of diagnostic value only when the cell inclusions are very easily recognized. Using this technic for comparative purposes on virous tissue not previously studied by other methods would only result in confusion and errors. For checking the presence of the crystals produced by Phaseolus virus 2, however, the crush technic was efficient since the cytological evidences of this virus are unusually stable. This method is very rapid, allowing free mingling of cell contents with trypan blue in a manner involving no permeability problems.

Simple free-hand sections were made to observe cytological virus evidence in petioles and stems. The parenchyma cells of stem sections proved very satisfactory for observation, but the attached trichomes were of no value in the case of these leguminous hosts.

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A MODIFIED TECHNIC FOR SALIVARY-GLAND CHROMOSOMES

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ABSTRACT.—**This technic was worked out for making permanent mounts of salivary-gland squashes of strains and species of Drosophilidae, which yield material difficult to work with. It includes a prior fixation in a modified Carnoy's fluid, staining in solutions of orcein in 60% or 70% acetic acid, part dehydration in the vapor and liquid of a 95% solution of alcohol, and direct mounting in Euparal by means of the drainage-method.**

During a study of the salivary-gland chromosomes of various strains of *Drosophila melanogaster* and other species of local Drosophilidae, the usual aceto-carmine technic of fixation staining, as recommended by Painter (1934), was followed, using an iron-aceto-carmine prepared according to the method of Belling (1926). While this technic yielded satisfactory results in the case of larvae which were fed on yeast-rich cultures at low temperatures, very variable and unsatisfactory results were obtained in the case of larvae from weak and slightly over-populated stocks, when the occasion arose to examine such, and also in the case of various species of the genus *Zaprionus*.

These variable results were ascribed to the staining capacity of the available batches of dye, and aceto-orcein was then tried out. For this dye also definite differences were found to exist in staining capacity in the case of the above-mentioned difficult stocks; ultimately the two best batches of the orcein available were selected, and these were, firstly, a British Drug Houses product obtained about fifteen years ago, and secondly, a product of Grübler obtained just prior to the war.

The various difficulties occurring with the larvae of certain weak strains and of the various local species of *Zaprionus* are: (1) The salivary-gland chromosomes do not stain very easily, or differentially, and (2) the chromosomes do not spread evenly during the squashing of the glands; they are soft and fragile, or tightly coiled.

The first difficulty has also been noted by Warters (1944), who modified the technic for certain groups showing varying affinities for the stain by a variation in the time used for staining and destaining. Both difficulties are also mentioned by Kodani (1939), who

ascribed the lack of proper staining capacity and the inability to render chromosomes flexible enough so they will spread out on crushing as due to a loss of fixing power of the acetic acid after being boiled with the dye; he advised a prior fixation with acetic acid before staining. A prior, or additional fixation, has also been recommended by various workers for the purpose of reducing the staining of the cytoplasm and thus increasing differential staining, and also for the hardening of the chromosomes: acetic acid for mitotic chromosomes by McClintock (1929), Carnoy's fluid for mitotic chromosomes by Koller (1940), and chloroform for the salivary-gland chromosomes of *Sciara* by La Cour (1941).

A prior fixation with Carnoy's fluid was utilized and found to improve the spreading of the chromosomes, to decrease the time of staining and increase the selectivity of the stain. The following technic for fixing, staining and spreading, and mounting salivary glands of difficult strains was then evolved. The method has provided uniformly good slides of salivary-gland chromosomes, and may be divided into its various procedures.

1. Prior Fixation. A preliminary fixation is given in a modified Carnoy's fluid (12 ml. absolute alcohol, 6 ml. chloroform, 1 ml. glacial acetic acid) as recommended by Koller (1940).

The glands were dissected out in Ringer and transferred on a flat-bladed dissecting needle to a drop of the fixing fluid on a well-slide. Up to certain limits the period of fixation varies; the longer the glands remain in the fluid, the greater is the extent of spreading attained during the crushing of the glands. The optimum time for the B.D.H. stain was determined as ¾–1 minute, and for the Grübler stain 1½–2 minutes.

2. Staining and Spreading. The stains found to give the most satisfactory results were the following: The B.D.H. and Grübler stains as 2% solutions in 70% acetic acid, made up by the method of La Cour (1941), and the Grübler stain in 60% acetic acid, made up and diluted as recommended by Demerec and Kaufmann (1945).

The glands are transferred from the fixing fluid to a few drops of the stain on a well-slide; the optimum period of staining was found to be 7–10 minutes for the B.D.H. stain and 5–7 minutes for the Grübler stains. It must, however, be noted that the time of staining varies for the different strains and species. The glands are then removed by means of a pipette to a fresh drop of stain on a clean slide and the cover glass is applied, the excess stain drained off, and a square of blotting paper laid over the cover glass.

The spreading of the chromosomes is affected by the amount of

pressure applied on the cover glass. Various workers use various methods, such as the ball of thumb, tapping with a blunt needle, or rolling with a glass rod. The following method has, however, been found to be the most satisfactory for the even spreading of the chromosomes, very often in the form of parallel arms: The square of blotting paper is lightly pressed to flatten the glands and remove excess stain, and a glass rod is then rolled firmly once across the paper. The amount of pressure exerted when rolling may be varied, so that varying degrees of spreading and stretching of the chromosomes may be attained.

3. Mounting. After trying out the method of McClintock (1929) for making aceto-carmine smears permanent, as well as the modified method recommended by Darlington and La Cour (1942), it was found that more simplified methods gave satisfactory results. For permanent mounts it seems advisable to spread, distribute evenly and dry a thin film of albumin on the slide, as described by Demerec and Kaufmann (1945).

Two methods of dehydration and mounting are utilized:

(a) The routine method is that recommended by Demerec and Kaufmann (1945). The slide is placed in a covered dish containing 95% alcohol so that the lower edge of the cover glass dips in the alcohol. After 6–12 hours the slide is immersed in 95% alcohol for 1–2 hours. The cover glass is then gently pried free from the slide while still in the alcohol, by inserting a sharp-pointed instrument beneath one edge, the excess alcohol is quickly drained off, a drop of Euparal is placed on the part of the slide containing the salivary glands, and the cover glass is applied.

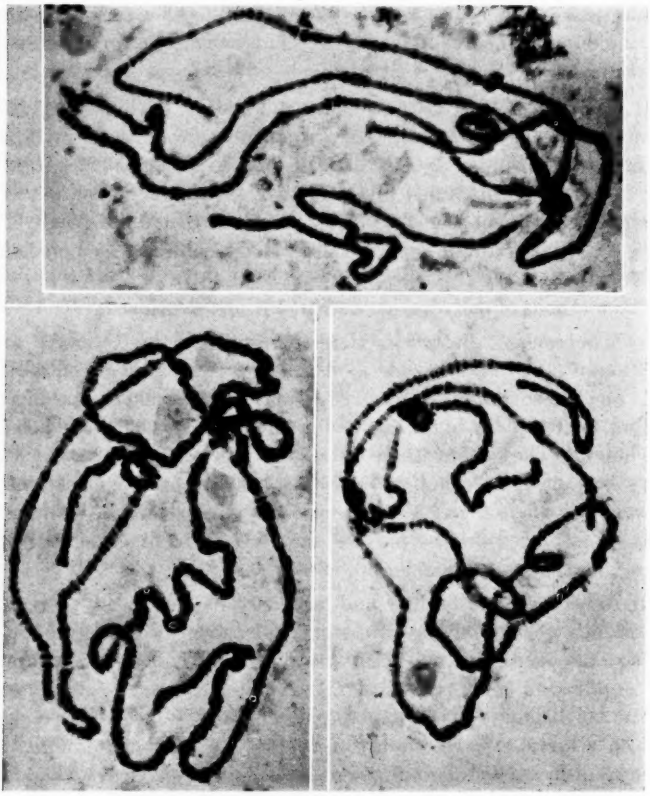
(b) Where especially fine and well-spread preparations are obtained, any accidental loss of chromosomes, due to disruption while lifting the cover glass, may be obviated by using the second method of dehydration and mounting. A modified version of the vapor method of Bridges (1937) for changing reagents is used. The slide is laid on a metal rack above the surface of 95% alcohol in a tightly covered dish. The edges of the cover glass are moistened with a drop of 70% alcohol and the slide is left for 24–48 hours in this dish with a differential vapor pressure.

After a displacement of liquids has occurred through evaporation and diffusion, the slide is immersed in 95% alcohol for 6–12 hours. It is then again placed on a metal rack above the surface of 95% alcohol in a covered dish, a square of blotting paper is placed along one edge of the cover glass and a drop of Euparal is applied along the opposite edge; the alcohol vapor in the atmosphere of the covered dish keeps

the Euparal in a liquid state, and by means of this drainage method the alcohol under the cover is replaced by Euparal.

This method has produced fine permanent preparations, however only if the Euparal rapidly and totally displaces the alcohol, since otherwise a fading takes place of the stain in the bands.

1



2 3

Salivary gland chromosomes stained by technic described. Fig. 1 (top), 2 (left) and 3 (right) as described in text.

Fig. 1–3 show nuclei of larvae from ordinary, slightly over-populated, laboratory stocks of *D. melanogaster*, i.e. without being prepared by means of special feeding and temperatures. The photomicrographs were taken through a Zeiss apochromat 20×, n.a. .65

and a compensating eyepiece 15×; the final magnification was ×450. Exposure was 2 seconds on Kodak Contrast Process Ortho, while the prints were D76 for 10 minutes. Fig. 1 and 2 are of nuclei stained with the B.D.H. orcein and mounted in Euparal by the drainage-method. Fig. 3 is of a nucleus stained with the Grübler stain and mounted by the routine method.

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BRILLIANT CRESYL BLUE AS A STAIN FOR CHROMOSOME SMEAR PREPARATIONS

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ABSTRACT.—**Brilliant cresyl blue is substituted for carmine in the acetocarmine technic. A mixture of one part of 1% toluidine blue and three parts 0.75% brilliant cresyl blue dissolved in 45% acetic acid gives good results. Propionic acid may be used instead of acetic acid for more rapid penetration.**

After several unsuccessful attempts to stain the salivary-gland chromosomes of *Drosophila macrospina macrospina* with acetocarmine, brilliant cresyl blue (a basic stain) was substituted for carmine and the chromatic discs were stained a dark blue. Brilliant cresyl blue is the stain commonly employed to show platelets and reticulated erythrocytes (Robertson, 1917). In the substitution for carmine in the acetocarmine, the brilliant cresyl blue is dissolved in 45% acetic acid and filtered. It is unnecessary to boil the stain.

The stain technic is as follows:

(1) Dissect out the salivary glands of the *Drosophila* larva in the usual manner (Painter, 1934).
(2) Place the glands in a small glass vial and add 5 drops of 0.75% brilliant cresyl blue[1] in 45% acetic acid.
(3) Stain for 30 to 45 minutes.
(4) Pour the contents of the vial onto a clean slide.
(5) Tilt slide to expose glands and blot off surrounding liquid.
(6) Break nuclei by tapping or pressing the cover slip.
(7) If overstained, introduce a drop of 45% acetic acid under the cover slip.
(8) Ring the cover slip with solution of Clarite dissolved in xylene.

In some cases good results were obtained if the glands were first fixed in 45% acetic acid and then placed in the stain. In several strains of *Drosophila macrospina macrospina* the salivary-gland chromosomes would stain if one part of 1% toluidine blue[2] were added to three parts of brilliant cresyl blue. It was also found that in some strains the salivary-gland chromosomes would be somewhat resistant to brilliant cresyl blue in acetic acid, but would stain more

[1]The lot used bore the certification No. NV–17.
[2]The lot used bore the certification No. NU–2.

readily if propionic acid were used instead of acetic acid. The Clarite-xylene ring dries fast and leaves an air-tight seal. Temporary slides will keep at least six months.

The advantages of this staining method are: (1) If salivary chromosomes resist the acetocarmine stain, the mixture of brilliant cresyl blue with acetic or propionic acid mixture may stain the chromatic bands. (2) There is no "luck" required to make a good staining solution as in acetocarmine (Smith, 1947). (3) Blue bands on a gray background are more distinct than those obtained by using acetocarmine.

The disadvantages are: (1) The mixture of acetic acid and brilliant cresyl blue is ink-like in appearance, and it is difficult to orient the gland in the stain. (2) A larger volume of stain is used, and hence the need of glass vials. There is a chance that loss of a gland may occur in transfer from vial to slide.

It appears that other basic dyes dissolved in acetic acid may be used in the rapid smear method to demonstrate chromosomes. Propionic acid may be used instead of acetic acid to facilitate penetration. Thus the research worker need not depend upon carmine or orcein, for he has a larger selection to help solve his staining problem.

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AN ACETO-CARMINE SQUASH TECHNIC FOR MATURE EMBRYO SACS

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ABSTRACT.—**A method is described by which whole embryo sacs of *Nicotiana*, *Petunia* and no doubt of certain other genera can be obtained readily in aceto-carmine ovule squashes. Although application of the technic to megagametogenesis and fertilization stages is stressed in this paper, use of the method allows development to be traced from the archespore up to the second or third division of endosperm nuclei. The success of the technic depends on four phases:—1) fixation in a medium that causes cell and nuclear structures to become pliable, yet rigid enough that their spatial relationships are not greatly distorted in squashing; 2) heat, which apparently increases the cohesion of cytoplasmic and nuclear constituents; 3) maceration to separate the embryo sac from surrounding cells; and 4) the use of a stain that differentiates the various nuclear structures as well as those of the cytoplasm. Staining of the cytoplasm, essential in some embryological investigations, is one advantage of the aceto-carmine squash method over the Feulgen procedure. In contrast to the Feulgen ovule squash method the aceto-carmine technic will probably be most useful in genera having numerous small ovules. Advantages and defects of the aceto-carmine procedure as compared with the paraffin technic are discussed, likewise the possible usefulness of the former in studies of sterility and in certain other special connections.**

Ovule squash technics based on the Feulgen reaction (Hillary, 1940) or combined with acetic-lacmoid (Darlington and La Cour, 1942) are known. Both methods are applicable chiefly to genera with large ovules, due to the difficulty of handling small ovules removed from the placenta. In the aceto-carmine technic, however, ovules are not separated from the placenta until ready to be squashed, and therefore the procedure is as adaptable to small as to large ovules. Actually small size is advantageous, since numerous minute ovules can be included in a single preparation. Although embryo-sac size has not been tested as a factor in success of the aceto-carmine technic, a certain maximum size may perhaps limit application of the method, as in the case of the Feulgen procedure (Hillary, 1940).

Since resting nuclei of the embryo sac stain only faintly with basic

[1]Contribution No. 119 from the University of California Botanical Garden.

fuchsin, the Feulgen method is valuable chiefly in studies of nuclear divisions. This disadvantage, plus the failure of cytoplasm to stain, causes the Feulgen technic, at least when used without counter-staining, to be relatively useless in many embryological investigations. With aceto-carmine, following the proper pre-treatment, these difficulties are not encountered. Without counter-staining, chromatin in all phases is well stained and differentiated from the cytoplasm, which is likewise stained to some extent; the positions of vacuoles and cytoplasmic strands can be made visible; and in fertilization stages the pollen tube cytoplasm is readily distinguished from that of the embryo sac.

This paper emphasizes application of the aceto-carmine method to stages from the 2-nucleate embryo sac through fertilization (i.e. stages impossible to find intact in aceto-carmine squashes unless special pre-treatment such as that described below is used). However, archespores, meiotic stages and changes occurring in the four megaspores can be detected in ovule squashes of *Nicotiana*. Thus, in this genus at least, the entire sequence from the archespore through megasporogenesis, megagametogenesis, fertilization and the first few divisions of endosperm nuclei can be followed. In genera in which the free-nuclear type of endosperm formation occurs, development might be traced even further.

Procedure

Since this technic may be useful to embryologists who are not familiar with squash methods in general, the procedure will be discussed in considerable detail.

Fixation. The fixative is 4 parts chloroform, 3 parts absolute ethyl alcohol and 1 part glacial acetic acid. Evacuation of air from ovaries immersed in the fixing fluid is not necessary. To the high chloroform content of the fixative is attributed the pliability of cell structures essential in keeping embryo sacs intact through the subsequent treatment. After the standard fixatives used in connection with aceto-carmine staining—alcohol-acetic (3:1) or alcohol-chloroform-acetic (6:3:1)—embryo sacs are so brittle that they are easily fragmented. In addition to flexibility, fixation of nuclear components superior to that of either 3:1 or 6:3:1 is also provided by the fixative.

The optimum fixation period extends from two days to three weeks. With less than 44–48 hours in the fixing fluid, embryo sac nuclear and cell membranes are not toughened enough to resist pressure applied in squashing. After approximately three weeks in the fixative, although

whole embryo sacs are obtained, the differentiation of nuclei and cytoplasm begins to deteriorate and chromosome structure is less sharply defined. Deterioration is slow, however, and material fixed in this formula may not be completely useless until more than a year has passed. (Fig. 1 to 4 were photographed from ovules fixed 3 months before being squashed; Fig. 5 to 10 from material squashed after 2 days to 3 weeks in the fixative.) Evidence obtained recently indicates that addition of chloroform to the vial of ovaries two or three days after fixation is begun, especially advisable if evaporation of the fixative has occurred, slows the deterioration process. Unlike tissues fixed in 3:1 or 6:3:1, those fixed in the formula given above shou'd *not* be transferred to 70% alcohol for storage, since alcohol causes the cytoplasm to darken and definition of nuclear structures to become inferior.

Heat and mordanting treatment. Several ovaries are removed from the fixing solution, drained on absorbent paper, and placed in a vial of 4% aqueous solution of iron alum. This vial and two vials of distilled water are placed in a water bath which is then heated rather rapidly to 75°C. The water bath is kept at that temperature for the following 7 minutes. After 3 minutes at 75°, ovaries are transferred to one of the vials of water; after 2 minutes they are changed to the other vial of water, in which they remain for another 2 minutes. The vial of ovaries is then removed from the bath and cold water is added to its contents to lower the temperature. After 2 or 3 minutes in cold water the ovaries are ready to be macerated. In the case of large ovules the hot iron alum and hot water treatments should perhaps be prolonged beyond the 7-minute total. Embryo sacs of some genera may respond more favorably to heat applied at a lower temperature (for example, 65°) and for a longer period of time (up to 1 hour in hot iron alum and an equal period of time in hot water).

The heat treatment apparently binds embryo sac constitutents together, enabling them to cohere through subsequent treatment. In addition to mordanting the stain, the iron alum protects nuclei from the action of hot water, as indicated by the fact that substitution of a 7-minute hot water treatment in place of the 7-minute heat treatment described above caused shrinkage of nuclear membranes and hydrolysis of chromatin. Other iron compounds might be more effective than iron alum. Solutions of iron acetate were found to be superior to iron alum for nuclear differentiation but inferior in preserving embryo sacs intact, probably because acetic acid was used as the solvent since iron acetate is practically insoluble in water. How-

ever, addition of 10 drops of a saturated solution of iron acetate in 45% acetic acid to 10 ml. of 4% aqueous solution of iron alum improves chromatin definition without detracting materially from embryo sac integrity. Results of controlled experiments showed that the improvement in definition was due to the iron acetate, not to the acetic acid in which it was dissolved.

Maceration. Ovaries are macerated in 50% hydrochloric acid for 10 minutes. Maceration of large ovules might be improved by longer immersion in the acid. Then they are washed in several changes of distilled water for a total period of at least 20 minutes, after which ovules are ready to be squashed. Differentiation of nuclei and cytoplasm usually is not harmed by soaking in water for several hours, and maceration is improved by it. However, the longer the tissues remain in water, the softer the embryo sacs become and the more delicate the squashing must be in order not to destroy their continuity. *Nicotiana* and *Petunia* ovules can be squashed successfully even after 2 hours in water.

Squashing and staining. An ovary is removed from the water and is drained on absorbent paper. While it drains, one or two ordinary dissecting needles from which the glaze has been removed are soaked in a drop of iron-aceto-carmine[2] on a slide, in order to add more iron to the stain; when the aceto-carmine begins to assume a slight purplish cast, enough iron has been added. (An abundance of iron is required to stain embryo sac nuclei immediately.) The ovary is placed in the drop of stain, the ovary wall removed with dissecting needles and ovules are scraped from the placenta into the stain. Fifty to one hundred ovules of the size found in *Nicotiana* species can be included in a single preparation to be covered by a 22×22 mm. cover slip. (Additional ovules may still adhere to the placentae and in case they are to be used for another preparation, the ovary may be transferred from the drop of stain to another slide; the ovules are sufficiently moist that they will not dry out for some time.) Bits of tissue other than ovules are removed from the stain. With a flat-bladed smearing implement[3] not constructed of iron, the ovules are

[2]Iron-aceto-carmine is prepared by refluxing for 4 hours 45% acetic acid with carmine crystals added in excess of the amount readily soluble in the quantity of acetic acid used. After refluxing, the stain is rapidly cooled, then filtered. To a small quantity (ca. 25 ml.) of the stock aceto-carmine thus prepared a saturated solution of iron acetate in 45% acetic acid is added; the amount of iron acetate that can be added without causing precipitation varies with the lot of stain, but is usually between 5 and 10 drops to 25 ml. of aceto-carmine.

[3]A toothbrush handle, with the end cut diagonally between upper and lower surfaces and the diagonal face polished, makes a useful squashing instrument.

tapped gently, never with a smearing motion, until the cells are well separated. The material is then distributed as evenly as possible over the area to be covered by the cover slip. As few motions as possible should be involved in separating or in spreading the cells; the less contact the embryo sacs have with the squashing instrument the more intact and the less distorted they will be. A cover slip is applied and the slide is heated over an alcohol flame, but the stain must not be allowed to boil. (Moderate heat swells cells and nuclei, improves spacing of nuclear components, intensifies staining of nuclei and decreases it in the cytoplasm.) The preparation is then examined[4] to determine whether the desired stages are represented, the stain sufficiently intense, and whether the embryo sacs require flattening. To intensify the stain the slide is alternately heated and cooled several times. To flatten and to spread the sacs, the slide is heated and while still hot is inserted between the two halves of a folded paper towel and pressure is applied to the cover slip region with the finger tips; lateral movement of the cover slip should be avoided. For study of nuclear structure and chromosome number, pressure is often desirable, although it may distort or blur outlines of embryo sac cells and positions of vacuoles.

The preparation may be sealed with a mixture of half gum mastic and half paraffin, applied to cover slip edges with a hot metal applicator. If permanent preparations are desired, any of several procedures may be used (Bridges, 1937; Hillary, 1938; La Cour, 1947; McClintock, 1929). A modification of Bridges' vapor method is being published by the author and has the advantage that the cover glass is not removed, thus possibility of loss or overlapping of desirable material in the finished preparation is eliminated (Bradley, 1948).

Destaining. Frequently the best preparations are temporary ones that have been overstained and are later destained. Destaining must be done with caution, however, since material usually cannot be restained after it has been destained. If material has been overstained initially or has become too dark with age, the sealing mixture is scraped away from two opposite edges of the cover slip and a mixture of *ca.* 5 parts 50% acetic acid and 1 part aceto-carmine is then drawn through the preparation by placing a drop of the mixture in contact with one open edge and absorbent paper in contact with the other. When the color of the stain taken up by the paper begins to appear pink or red, rather than purplish, no more liquid should be drawn through, even though cells in the preparation are still too dark,

[4] In any microscope studies of aceto-carmine preparations, a blue-green light filter is invaluable.

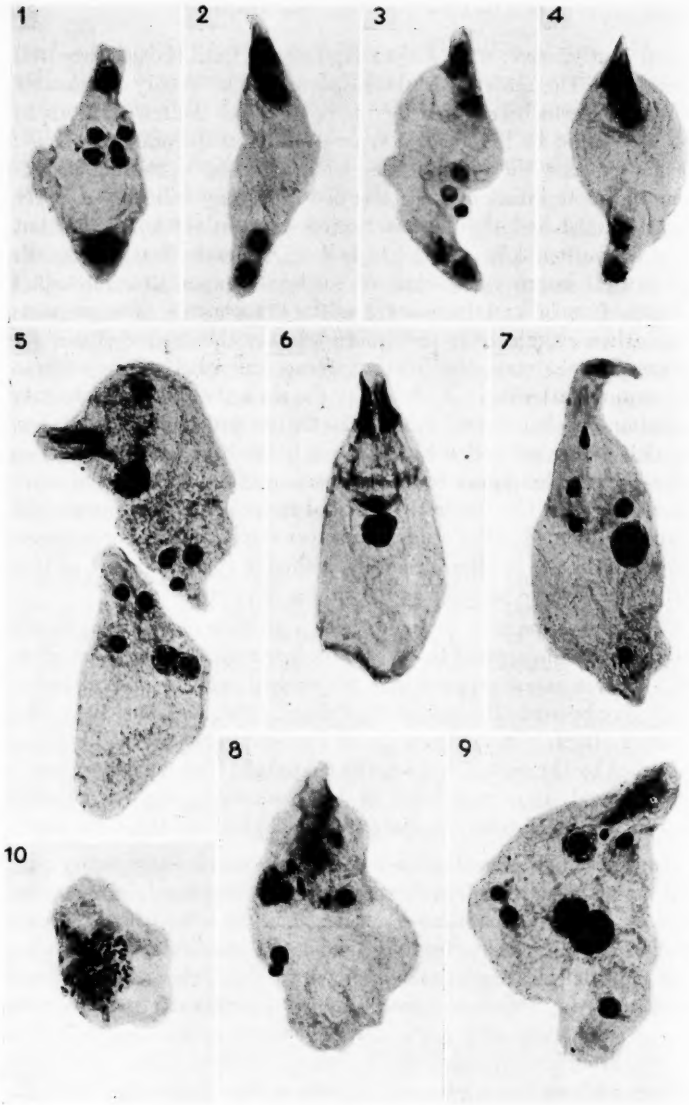

PLATE 1

All photomicrographs are of embryo sacs of *Nicotiana tabacum* and were selected more for nuclear phase or structure or for fertilization stage than for cytoplasmic features. The micropylar end is uppermost in all sacs. Somatic cells around the embryo sacs were blocked out, but nothing within the sac boundaries was retouched. All figures $\times 275$. Description of figures on p. 35.

1. Prophase of the second division. Note the dense cytoplasm around the nuclei and the large vacuole between them, part of which is overlaid by somatic cells.

2. Prophase of the third division. Some of the lines in the central region are due to crumpling of the sac wall. The same applies to Fig. 1, 3 and 4.

3. Metaphase of the third division. The sac was badly distorted in squashing, and somatic cells overlap part of it. The end regions have not been damaged and show the different planes of division.

4. Anaphase of the third division. The two chromosome groups in each of the terminal division figures are partially or totally superimposed due to the planes of division. Note the greater cytoplasmic density and more pointed outline of the micropylar end, which appear also in Fig. 2 and 3.

5. Two mature embryo sacs. Cell outlines are blurred due to pressure on the cover slip. In the more distorted, upper embryo sac the three antipodals are seen, also the fusion nucleus, egg and synergid nuclei; the egg cell outline is vaguely indicated. In the lower sac no antipodals are present (see text); a ring of relatively dense cytoplasm is seen around the egg nucleus; the polar nuclei appear to be fusing; synergid cells are distorted but the outline of the one to the right is dimly represented; two darker lines along the synergid apices are remnants of the refringent outer wall of the megaspores (see text) and are seen also in Fig. 2 and 6.

6. A mature embryo sac. The large central nucleus is probably not the fusion nucleus but rather the single polar nucleus formed in this sac, judging by the size of its nucleolus (cf. Fig. 5 and 7) and by the types of abnormalities found in this species. If that is the case, the first division failed, which also accounts for absence of antipodals. The outline of the egg cell up to the dense cytoplasmic region of the synergids may be traced, and with less certainty the basal outlines of synergid cells, slightly above the egg nucleus and superposed by the egg cell. Internal nuclear structure of the synergids is absent because the nuclei are becoming pycnotic. Positions of starch grains are indicated by pale circular areas surrounded by darker lines of cytoplasm and are seen also in Fig. 9.

7. Pre-fertilization. The pollen tube has entered the embryo sac and the sperms are seen as two small curved nuclei at the base of the pollen tube cytoplasm. The nucleus uppermost in the sac, apparently lying in the cytoplasm of the pollen tube, is the degenerating synergid nucleus. (The nucleus of the other synergid is missing, probably due to the squashing.) Between the synergid nucleus and the sperms is the degenerating vegetative nucleus of the pollen tube. To the right of the sperms is the egg nucleus, somewhat out of focus, and below that is the large fusion nucleus. Near the base of the sac is the single antipodal.

8. Pre-fertilization, somewhat more advanced than in Fig. 7. At the lower left, overlapping the sac, are two somatic nuclei. Above is the single polar nucleus, and the small elongated nucleus to the right of it is a sperm. The other sperm nucleus, slightly curved, is to the left of the egg nucleus and appears to have entered the egg cell. The irregularly shaped chromatin body to the upper right of the polar nucleus is the degenerating vegetative nucleus. Appearing to lie in the extremely dark pollen tube cytoplasm, but actually superposed by it, is the nucleus of the undamaged synergid. To the upper right of that is the pycnotic nucleus of the synergid ruptured by the pollen tube.

9. Fertilization—an advanced stage. The uppermost chromatin mass, near the base of the darkly stained pollen tube cytoplasm, is the degenerating synergid nucleus. Just below it is the pycnotic vegetative nucleus, and below that a "nucleus" that is actually the fusing egg and sperm nuclei, surrounded by the deeply stained cytoplasmic structures characteristic of late fertilization and zygote stages. The larger nucleolus, contributed by the egg, is left of center of the fusing nuclei and the dim outline of the sperm appears toward the right. The intact synergid is to the right of the vegetative nucleus; the lower part of its cell outline is visible. Apparently the second sperm has merged with one of the polar nuclei prior to their complete fusion. To the left of the polar nuclei are two antipodals that have migrated from the chalazal end of the sac, a common occurrence in this species. The third antipodal is seen below the polar nuclei.

10. Metaphase of one of the early endosperm nuclear divisions. Although some of the chromosomes are not in good focus, all 72 can be counted. The haploid number of *N. tabacum* is 24.

since the excess acid will continue to destain for a time. Any liquid left beside the cover slip should be blotted up before fresh sealing mixture is applied to *all* edges of the cover slip.

Summary of Procedure

(1) Fix ovaries for at least 2 days in 4 parts chloroform: 3 parts absolute alcohol: 1 part glacial acetic acid.
(2) Drain ovaries and place in a vial of 4% aqueous iron alum.
(3) Place vial and 2 vials of distilled water in a water bath, heat bath to 75°C., and keep at that temperature for 7 minutes.
(4) Leave ovaries in iron alum at 75° for 3 minutes.
(5) Transfer to vial of water—2 minutes at 75°.
(6) Change to other vial of water—2 minutes at 75°.
(7) Remove vial of ovaries from the bath and pour cold water into the vial. Leave ovaries in cold water for 2–3 minutes.
(8) Macerate in 50% hydrochloric acid—10 minutes.
(9) Wash in several changes of distilled water—at least 20 minutes in water, altogether.
(10) Drain an ovary and place in a drop of iron-aceto-carmine on a slide. Scrape ovules from the placentae into the stain. Remove bits of tissue other than ovules from the stain.
(11) Tap the ovules gently with a flat-bladed squashing implement until the cells are separated.
(12) Apply a cover slip and heat the slide over an alcohol flame, without boiling the stain.
(13) Examine the preparation to see whether pressure and/or more heating is required.
(14) When the preparation is adequately stained, seal it or make it permanent.

Discussion

Although many more somatic cells than embryo sacs are present in ovule squashes, a considerable number of embryo sacs in good condition are to be found in a successful preparation. Some sacs are unavoidably distorted, broken or covered by somatic cells, but an average squash may contain much more usable material than an average paraffin section slide; [2- and 4-nucleate sacs are most susceptible to crumpling of cell walls during the squash procedure, because of their large central vacuoles (cf. Fig. 1–4 with 5–9)]; 2- to 8-nucleate sacs are easily recognized by their size and the characteristic appearance of their nuclei. Earlier stages, although not as readily distinguished from somatic cells on the basis of cell size, can be identified by cell shape and the staining quality and size of their nuclei.

Compared with the paraffin technic, the aceto-carmine squash method for embryo sacs has certain advantages and also some disadvantages. Among the former are: 1) great reduction in time and labor involved; 2) superiority in showing (a) nuclear differentiation, and number and morphology of chromosomes and (b) changes in

sperm and "x-body" structure from time of penetration of the pollen tube into the ovule to post-fertilization stages (cf. Goodspeed, 1947); 3) absence of the uncertainty of interpretation encountered in studies of paraffin sections when the embryo sac must be reconstructed from two or more sections; and 4) adaptability of squashed embryo sacs to photomicrography (Plate 1). On the other hand, the development of nucellus, integuments, etc. in relation to embryo sac development cannot be followed in squash preparations. Also, orientation of megaspores and embryo sacs in relation to chalazal and micropylar ends of the ovule may be lost in some genera. In *Nicotiana* and *Petunia*, however, orientation is possible even in mature sacs, due to retention at their synergid apices of traces of a peculiar refringent wall that had earlier surrounded meiotic and megaspore cell groups. (Fig. 2, 5 and 6). A third defect of the squash technic would apply only to genera in which the various embryo sac cells are separated by walls that include membranes subject to action of the macerating fluid, in which case the cells would be separated.

Some types of abnormal embryo sac development can be detected with much greater certainty in squash preparations than in paraffin sections. For example, in *Nicotiana tabacum* different mature embryo sacs from the same ovary showed variation in number of antipodals; some had three, others one, and in still others none was present (Fig. 5 to 9). Studies of earlier stages showed that failure of one or two nuclear divisions was the cause. In paraffin sections such differences in number of antipodals might have been interpreted as loss of one or more nuclei in sectioning. Other abnormalities seen in squash preparations included embryo sacs with supernumerary nuclei, frequently due to extra divisions of antipodals and in some cases to divisions of other nuclei. Several instances of twin embryo sacs were seen, probably of the type developing within a single nucellus, since the two sacs remained attached through the squashing process. In addition, a few cases of multiple pollen tubes in embryo sacs were found in *Nicotiana longiflora* (Goodspeed, 1947).

In genera in which the aceto-carmine squash method is successful, studies of the cytology of sterility may be facilitated by its use. Pycnosis of nuclei should be a clue to the stage of megasporogenesis, megagametogenesis or early post-fertilization beyond which development does not proceed. Rather early stages of nuclear degeneration are identified more readily in aceto-carmine squashes than in paraffin sections stained with hematoxylin. (Examples of degenerating nuclei, not connected with sterility, however, are seen in Fig. 6 to 9. Also cf. illustrations, Goodspeed, 1947.) Another type of sterility, not due to nuclear and cell degeneration, has been detected in ovule squashes of several *Nicotiana* species in which no seed was setting early in the flowering season—a common phenomenon in many genera. In ovaries fixed at least 48 hours after anthesis, fully differentiated 8-nucleate embryo sacs were rare and in many only 2- and 4-nucleate sacs were seen. In most preparations no pollen tubes were found; in a few, the occasional tube seen had not penetrated an embryo sac. Lack of synchronization in floral development is no doubt a factor in sterility here—perhaps the only factor. Unfor-

tunately ability of pollen to germinate was not investigated, but since immaturity of ovules probably implies a similar state in stigma and style, it may be assumed that stigma conditions were not appropriate for pollen germination.[5]

Through use of the aceto-carmine squash method certain problems long discussed by embryologists can be solved or at least brought nearer solution. One is the nature of "x-bodies." From observations of fertilization stages in ovule squashes and of vegetative nucleus phases in stigma, pollen tube[5] and ovule squashes, no doubt remains that in *Nicotiana* and *Petunia* one of the "x-bodies" is the degenerating vegetative nucleus and the other the degenerating nucleus of the synergid damaged by penetration of the pollen tube into the sac (Fig. 7, 8 and 9. Cf. also Goodspeed, 1947). In aceto-carmine squashes there is no possibility of confusing either of these two degenerating nuclei with cytoplasmic structures. Although the Feulgen stain is more specific for chromatin, staining only structures containing desoxyribose-nucleic acid when skillfully applied, aceto-carmine is almost as useful in differentiating nuclei (either healthy or pycnotic) from cytoplasmic constituents. Only in certain cases (tannins, or contents of abnormal cells or pollen tubes) are cytoplasmic granules stained by aceto-carmine with the same intensity as the nucleic-acid-containing chromatin and nucleoli. Usually, deeply stained cytoplasmic particles can be distinguished from nuclear elements by differences in refringence, and often the former can be made to disappear from view by opening wide the substage diaphragm, while the latter remain visible.

Certain evidence in regard to cellular structure of embryo sac components is obtainable from squash preparations. In *Nicotiana* and *Petunia* the outlines of egg, synergid and antipodal cells and also the disposition of cytoplasmic regions of different densities within the cells are frequently clear (Fig. 6, outline of egg cell. Cf. also illustrations, Goodspeed, 1947). In some cases definite membranes are apparent, separating the cells from one another and from the central region containing the polar nuclei or fusion nucleus. That the membranes are not subject to hydrochloric acid action, (at least to a visually detectable degree) and that the cells are not held together merely by the wall surrounding the entire sac is indicated by

[5]In this connection, a simple method of preparing material for investigation of pollen germination and pollen tube development *under natural conditions* (including growth rate, generative nucleus division, sperm and vegetative nuclear structure, etc.) involves only the following few steps: fixation of stigmas and styles in the formula given for ovule squashes, maceration of the stigma in hydrochloric acid (50% for 10 minutes) before squashing it in iron-aceto-carmine; scraping out of pollen tubes from the interior of successive regions of style that have been slit lengthwise and the cut edges folded back, teasing apart of pollen tubes in a drop of iron-aceto-carmine; adding a cover glass, heating the slide, whether it holds pollen tubes from the style or macerated stigma cells and pollen grains, and applying pressure to the cover glass if necessary. To stain the vegetative nucleus a large amount of iron in the stain may be required, likewise several alternations of heating and cooling.

the manner in which embryo sacs are disrupted by extreme pressure. Breakage is almost invariably in the central region, and remnants of the sac include parts of the central "cell" plus either the intact egg apparatus or the group of antipodals, neither of which shows any tendency toward separation of its constituent cells, even though the pressure applied was sufficient to spread them considerably. Failure of the membranes to be weakened by hydrochloric maceration suggests that they may be similar in structure to membranes between recently formed somatic sister cells. Such sister cells usually remain together as a unit after maceration, although the membrane separating them must have been developing during the time required for their nuclei to progress from anaphase-telophase to a moderately advanced resting stage. (Sister cells can be recognized by their identical chromatin state, cell and nuclear size and by the mirror-image positions of their nucleoli.) Incomplete formation of the membranes—incomplete either in chemical changes leading to formation of pectic substances subject to hydrochloric action, or in the continuity of such pectin molecules—is probably responsible for failure of somatic sister cells to separate after maceration, and the same may be true in regard to embryo sac cells.

Although the aceto-carmine method has been tested chiefly on *Nicotiana* species and one *Petunia* species, all of which have given satisfactory results, it was tried also on two or three ovaries of one species of each of the following genera: *Scilla*, *Aloe*, *Aeonium*, *Papaver*, and *Aquilegia*. In the case of the two monocots, *Scilla* and *Aloe*, whole embryo sacs were not obtained, because a group of cells at the chalazal end of the embryo sac could not be macerated with hydrochloric acid and attempts to separate it from the sac resulted in rupturing of the sac. In *Aeonium* the method was partially successful and with changes in heat or maceration schedules would probably yield satisfactory results; the embryo sac of *Aeonium* is contained in a thick wall which ruptures in some cases to allow cytoplasm and nuclei to flow out intact. In *Aquilegia* and *Papaver* preparations complete 8-nucleate sacs were found, and only minor variation, if any, of the method should be necessary. From these investigations it may be predicted that the technic described here will be most successful in genera having many small ovules, more commonly occurring in some dicot families than in those of monocots. The obstacles to maceration presented in *Scilla* and *Aloe* will perhaps be encountered also in genera related to them. However, a macerating agent might be found that would permit the aceto-carmine squash procedure to be adapted to such genera. Probably in the Solanaceae in general, and perhaps in related families as well, no modification of the technic will be required.

It is hoped that other investigators will develop variations of the aceto-carmine squash method that will extend its use to studies of embryo sac development in genera that will not respond favorably to the method given here.

The author wishes to express appreciation to Dr. T. H. Goodspeed for providing the opportunity to develop the technic described.

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A METHOD FOR MAKING ACETO-CARMINE SQUASHES PERMANENT WITHOUT REMOVAL OF THE COVER SLIP[1]

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ABSTRACT.—**Two modifications of Bridges' alcohol vapor method for making smear and squash preparations permanent are described. The variation of more general significance permits euparal to be applied without removal of the cover slip. Thus, possibility of loss, distortion or overlapping of desirable material is eliminated from this phase of preparation. The second modification is a reduction in duration of the alcohol vapor treatment. Although the latter alteration in schedule has no particular value in connection with material of many genera, it allows the vapor method to be applied to tissues of *Nicotiana* and may be useful in dealing with material of some other genera.**

To supplement a technic for obtaining whole embryo sacs in aceto-carmine squashes of *Nicotiana* ovules (Bradley, 1948), a method was required that would enable preparations to be made permanent without removal of the cover slip. Since ovule squashes contain a preponderance of somatic cells, removal and replacement of the cover slip, as in the procedures commonly used, frequently cause superposition of embryo sacs and somatic cells, in addition to the loss of valuable material and the distortion of cell peripheries liable to occur in any preparation when the cover slip is removed.

Bridges' (1937) alcohol vapor method for making smear and squash preparations permanent had hitherto been unsuccessful when applied to tissues of *Nicotiana* species, because of excessive darkening of the cytoplasm. However, since Bridges' method offered the best possibilities for obviating cover slip removal, variations were sought, and found, to adapt that technic to the requirements.

The modifications reported here are applicable to aceto-carmine squashes of anthers and root tips as well as to those of ovules. Although they have not been tested on material fixed and stained by other technics, it is probable that they can be applied after any pro-

[1]Contribution No. 120 from the University of California Botanical Garden.

cedure that has been followed successfully by the original schedule of the Bridges method.[2]

If permanent preparations are to be made of tissues fixed in a formula having a high chloroform content, such as the one used for ovule squashes (Bradley, 1948), as much of the excess fixative as possible should be absorbed from the material with filter paper before post-fixation steps in preparation are begun.[3] Otherwise, chloroform is carried into the stain and may not be replaced completely by alcohol in the subsequent vapor treatment. Since chloroform is not readily miscible with euparal, aggregates of refringent granules will form when euparal is applied. Anthers, or small buds from which anthers are to be removed for squashing, should be drained with particular thoroughness, since no treatment with fluids (during which some chloroform would be removed) intervenes between fixation and squashing.

The technic can be applied either to freshly made preparations or to aged temporary ones. In the case of temporary preparations sealed with a mixture such as gum mastic and paraffin, as much of the seal as possible is carefully scraped away from both slide and cover slip. A drop of xylene is spread over the areas formerly covered by the seal and is then blotted up with lens paper, special care being taken to remove xylene and dissolved seal from the line of union of cover slip edges with the slide.

Before a preparation is made permanent, it may require destaining, particularly if the stain contains an abundance of iron or if the preparation is an old temporary one. (Frequently, however, superior differentiation is found in overstained preparations after they are destained.) In destaining, loss of material is reduced if the slide was heated and pressure applied to the cover glass when the preparation was made. To destain, a drop of dilute aceto-carmine (*ca.* 1 part stain to 5 or 6 parts 50% acetic acid) is placed on the slide and allowed to run around the cover slip and underneath it. The slide is warmed over an alcohol flame (the stain must not be allowed to boil) to speed penetration of the dilute stain to tissues under the center of the cover

[2]After this paper was submitted for publication, the method was tested on Feulgen squashes of *Vicia* root tips and *Rhoeo* and *Agapanthus* anthers and proved successful in each case.

[3]Subsequent investigation has shown that excess chloroform can be removed more thoroughly by soaking the tissue in water for 10 to 20 minutes before it is stained. This is particularly advisable when the material is to be stained with aceto-carmine without preliminary maceration or when the Feulgen procedure is to be used. In the latter case the chief purpose of chloroform removal is to increase the intensity of the stain, since chloroform apparently interferes with hydrolysis or staining with basic fuchsin, or both.

slip. Then the slide is placed within the fold of a paper towel and the region over the cover slip is pressed gently to squeeze out excess liquid. The acetic acid will continue to destain for a time, but if, in view of that, the cytoplasm is still too dark, the above procedure can be repeated. The degree of destaining is rarely equal in all parts of the preparation, due to differences in density of material and in the rapidity with which the destaining fluid comes in contact with tissues in different regions.

Before the preparation is mounted in euparal, it must be dehydrated. This is accomplished, as in the Bridges' method, by vapor exchange. The slide is placed on end in a jar that can be covered tightly, the sides and bottom of which are lined with paper towels saturated with 95% ethyl alcohol. The period of time in alcohol vapor varies with the material. Bridges recommended 24 hours or longer, and this schedule is successful with tissues of many genera. However, material of *Nicotiana* species (and perhaps that of certain other genera) requires a much shorter treatment; 4 to 6 hours' treatment is sufficient, and a 24-hour period in alcohol vapor causes the cytoplasm to darken to the extent that preparations become useless.

After an appropriate period of time in the vapor jar, the slide is removed, and to insure complete exchange of fluids, a few drops of absolute alcohol are applied around the cover slip edge, allowed to run underneath, then pressed out. Several drops of diaphane or euparal, diluted with absolute alcohol to the consistency of thin cream are spread around the edges of the cover slip. The slide is placed, cover slip up, on a piece of absorbent paper in a flat, covered container. The paper is moistened with a small amount of absolute alcohol—enough to soften the euparal and allow it to infiltrate the tissue without diluting it to the extent that it flows over the cover slip. If the container is a $10 \times 10 \times 1.5$ cm. petri dish, 10 to 15 drops of alcohol are sufficient.

After approximately 24 hours the slide may be removed. It should be kept at room temperature for 3 or 4 weeks to allow the euparal to dry slowly, since at higher temperatures the rapidly evaporating alcohol may be replaced by air. In case air bubbles should form under the cover slip, the slide may be returned to the second alcohol vapor chamber until the euparal has softened. Then, by locally directed pressure on the appropriate cover slip regions, the air can be forced out to be replaced by euparal.

Summary of Procedure

(1) If the preparation is a sealed temporary one, scrape away the seal and remove remaining traces of it with xylene.

(2) If necessary, destain either freshly made or previously sealed preparations with 5 or 6 parts 50% acetic acid plus 1 part aceto-carmine.

(3) Place the slide on end in an alcohol-vapor jar and leave it there for 4 to 6 hours.

(4) Allow a few drops of absolute alcohol to run underneath the cover slip, then press out the alcohol.

(5) Apply euparal or diaphane all around the edge of the cover slip.

(6) Place the slide, cover slip up, in an alcohol-vapor jar in which the atmosphere is *not* alcohol-saturated. (A 10×10×1.5 cm. petri dish requires not more than 10 to 15 drops of absolute alcohol.) After *ca.* 24 hours the slide may be removed.

(7) Allow the euparal to harden at room temperature for three or four weeks.

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LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

MICROSCOPE AND OTHER APPARATUS

OETTLE, A. G. **A technique for changing from a bright to a dark ground condenser while a slide bearing a watery mount is still under an oil immersion objective.** *J. Roy. Micr. Soc.*, **64**, 124–8. 1944.

The apparatus consists of a brilliant source of light with an iris diaphragm, a wide aperture reflecting dark-ground condenser such as the cardioid, and an oil immersion objective with an iris diaphragm. The condenser should produce a hollow cone of light where the most oblique rays have a numerical aperture greater than 1.33 and the least oblique rays have a numerical aperture less than the maximum for the objective. Theoretical requirements for this system are fulfilled by the Zeiss-Siedentopf cardioid condenser N. A. 1.21–1.37 and a Zeiss achromatic oil immersion objective rated as 2 mm. (90×), N. A. 1.25, with an iris diaphragm by which the N. A. may be reduced to 0.55.

Good quality slides are essential. Four steps in the technic are given. With this rapid technic changing from bright to dark ground, illumination is accomplished without disturbing the slide under oil immersion.—*Paul G. Roofe.*

PHOTOMICROGRAPHY

FROOME, K. D., and JARRET, B. A. **The instantaneous photomicrography of living micro-organisms.** *J. Roy Micr. Soc.*, **64**, 136–46. 1944.

The camera, with its shutter and body, mechanical timer, electric timer and electric shutter completely described in this article, overcomes the difficulties occurring in the conventional micro-cameras. The following new features are installed in the camera to overcome these drawbacks: (1) Ciné-film is used decreasing the exposure time, thus eliminating blurring by a rapidly moving object. (2) The principal feature of their camera lies in the shutter, which is electrically operated, the timer, both mechanical and electrical, being separate from the camera, thus eliminating vibration by the old methods. (3) The timer is released by foot, thus allowing free use of both hands. (Shutters of normal design were hand operated.) (4) Maximal light reaches the film because the shutter itself is a partial mirror. By inclining the shutter a convenient percentage of light is reflected into an auxiliary viewing eye piece up to the instant of exposure. Exposure time is thus cut down.

Applications of the camera are given. The chief value lies in the comparative ease with which it is possible to take photographs of moving microscopic life.—*Paul G. Roofe.*

RAEBURN, L. T. **Side focusing eyepiece for small micro-cameras.** *J. Roy. Micr. Soc.*, **64**, 147–8. 1944.

A concise description is given, illustrated by two figures, of a camera with a built-in side focusing eyepiece. By the method described, the following difficulties are overcome: (1) rigidity of the apparatus with its vibration; (2) the necessity of focusing on ground glass or plane glass by means of a hand magnifier.

The box attachment to the camera is of such a nature that by merely swinging a mirror (housed in the box) to a vertical position, one is able to proceed with the taking of the photograph.—*Paul G. Roofe.*

MICROTECHNIC IN GENERAL

BARGHOORN, E. S. **Use of phenol formaldehyde and vinyl resins in sealing liquid mounting media on microscope slides.** *Science*, **106**, 299–300. 1947.

A method is outlined for using phenol formaldehyde and vinyl resins for sealing glycerol and lactic preparations on microscope slides, the former being indicated as superior to sealing compounds now in use. Details for thinning, application of the method and results of aging are described.—*T. M. McMillion.*

HARTLEY, W. G. **A variable phase-contrast system for microscopy.** *Nature*, **159**, 880–1. 1947.

The author describes a variable phase-contrast system based on refracted and dioptric rays which are polarized at right angles to each other. A path difference is introduced by passing these rays through a bi-refringent screen, which produces the effect of different optical thicknesses because its refractive index is different for two perpendicularly polarized rays.—*C. Randall.*

VAN CLEAVE, HARLEY J., and ROSS, JEAN A. **Use of trisodium phosphate in microscopical technique.** *Science*, **106**, 194. 1947.

The author discusses the use of dilute Na_3PO_4 solution, usually 0.25%, for softening and increasing the permeability of dried or resistant material. General discussion of the use of the solution on acanthocephalans preliminary to staining and mounting is given, with indications that the reagent is superior to other detergents.—*T. M. McMillion.*

ANIMAL MICROTECHNIC

ASH, J. E. **Technical methods in use at the Army Institute of Pathology.** *J. Tech. Methods*, **26**, 1–59. 1946.

The entire issue is devoted to a presentation of the details of all technics used in the preparation of tissue at the Army Institute of Pathology. General procedures for fixing, embedding, and staining tissues are described plus many special technics for particular tissues. Staining methods for connective and nerve tissues, microorganisms, blood, and cell products are given. Also included is a section on the preparation of museum specimens.—*C. Randall.*

CALLAN, H. G., and MONTALENTI, G. **Chiasma interference in mosquitos.** *J. Genetics*, **48**, 119–34. 1947.

Satisfactory preparations of testes of mosquito larvae or pupae were obtained as follows: Dissect out under a binocular microscope; fix for a few minutes in a mixture of 3 parts absolute alcohol to 1 part galcial acetic acid; stain for about 5 min. in iron aceto-carmine; squash lightly under a cover slip.—*V. Karanagh.*

CHU, C. H. U. **Staining of nerve endings in mouse epidermis by Feulgen's nucleal reaction.** *Science*, **106**, 70. 1947.

An easier and more reliable method for staining nerve endings in mouse epidermis by Feulgen's nucleal reaction is described. The essential technics employed are those in regular use for other purposes, reference being made to standard handbooks of microscopic technic. Reference is also made to staining nerve fibers by Schiff's reaction.—*T. M. McMillion.*

CLARK, A. E. **A method for staining grossly fatty tissues with Scharlach R.** *J. Path. & Bact.*, **59**, 337–8. 1947.

To avoid bothersome stickiness of frozen sections from grossly fatty kidney and liver, gelatin embedding was found efficacious in allowing the preparation of flat sections. The following procedure was used: After formalin fixation, wash tissue in running water 24 hr. Soak in 12.5% aqueous gelatin at 37°C. for 24 hr.; transfer to 25% gelatin for another 24 hr. Embed in 25% gelatin and refrigerate to set. Place the block in 5% formalin for 24 hr. Trim the gelatin block to within 1 mm. or so of the tissue and cut sections 10–15μ on the freezing microtome. Transfer sections from knife with a soft brush or with a finger moistened with 50% alcohol to a dish containing 50% alcohol. Transfer the sections to 0.5% gelatin for a few seconds. Pick up sections from the gelatin solution on clean glass slides; a finely drawn glass rod is useful. Drain off excess solution but do not let sections dry.

Place slides in a staining trough over formalin at 37°C. for at least 1 hr. The

slides must not rest in the formalin. Immerse the slides in dilute formalin and leave until ready to stain.

Staining: Rinse sections in distilled water. Transfer to 70% alcohol. Stain in Scharlach R (saturated solution Sudan IV in equal parts of alcohol and acetone) for 3–5 min. Rinse thoroughly in 70% alcohol, then in distilled water. Stain in Ehrlich's hematoxylin for 10 min. Place in running water till blue. Differentiate in acid alcohol. Treat again in running water.

Mount in glycerol jelly or in G. W. Moore's modification of Farrant's mounting medium, composed of: picked gum arabic, 100 g.; cane sugar, 50 g.; distilled water, 300 ml.; thymol, 0.2 g.; glycerol, 25 ml. Dissolve the gum arabic and cane sugar by steaming. Cool, clear with the white of 2 eggs and filter through muslin. Add thymol and finally the glycerol.—*S. H. Hutner.*

CROOKE, A. C. **A rapid supra-vital staining method for assessing the viability of human spermatozoa.** *Nature*, **159**, 749. 1947.

The following method may be used for supra-vital staining of human spermatozoa: Place 4 or 5 drops of seminal fluid on a clean slide, add one drop of stain and mix well; let stand 2–3 min.; withdraw a drop and make a thin smear on a clean slide; let dry in air and then fix for 15–20 sec.; dip slide in alcoholic iodine and into 90% alcohol; allow to dry; counterstain 15–30 sec.; rinse in water and differentiate with care in 90% alcohol.

The reagents are prepared as follows:

Supra-vital stain: 50 ml. distilled water; 1 g. Revector soluble blue 706; 1.5 g. glucose; 0.1 g. NaCl; 0.3 g. Na_2HPi_4, hydrated; 0.005 g. KH_2PO_4. This solution must be made up fresh or stored in 1 ml. ampules and autoclaved.

Counterstain: 1% neutral red in distilled water.

Fixative: 1 vol. saturated solution of $HgCl_2$ in distilled water; 1 vol. absolute alcohol; followed by 90% alcohol containing a few drops of iodine in KI to give a straw-colored solution.

The nuclei of the spermatozoa which were dead when the seminal fluid was mixed with the stain are blue or purple; those that were alive are clear red, and a few non-nucleated forms remain unstained.—*C. Randall.*

GOLDSCHMIDT, ELIZABETH. **Studies on the synapsis in salivary chromosomes of hybrid Chironomus larvae.** *J. Genetics*, **48**, 194–205. 1947.

Salivary glands of prepupae were dissected out in a drop of Ringer's solution, left for 10 min. in 45% acetic acid, and stained 10 min. in aceto-carmine before crushing. Permanent preparations were obtained by transferring the smears through 95% alcohol into Venetian turpentine.—*V. Kavanagh.*

R. D. LILLIE. **Reticulum staining with Schiff reagent after oxidation by acidified sodium periodate.** *J. Lab. & Clin. Med.*, **32**, 910–12. 1947.

The following procedure is recommended: Deparaffinize thin sections (5μ or less) and bring up to 100% alcohol as usual. Soak 5 min. in 1% collodion in 50:50 ether and alcohol, drain and harden 5 min. or longer in 80% alcohol. Wash 5 min. in running water and immerse in sodium periodate (Na_3IO_5 1.0 g., 70% HNO_3 0.5 ml., and distilled water 100 ml.) for 10 min. Wash 5 min. in running water. Immerse 15 min. in Schiff reagent (place 1 g. basic fuchsin in 100 ml. of distilled water at 95°C., filter, cool and add 2 g. $HNaSO_3$ and 20 ml. of *N*/1 HCl). Transfer to 3 successive baths of 0.5% $NaHSO_3$. Wash 10 min. in running water. Stain 5 min. in Weigert's iron hematoxylin. Counterstain 1 min. in saturated picric acid or 1% orange G. Dehydrate with alcohols and clear with xylene. All reticulum, subepithelial basement membrane and the stroma of cerebral capillaries stain bright purplish red. Collagen stains pink. Cartilage matrix stains red-purple, epithelial mucin reddish purple to almost violet, and glycogen dark red purple to almost black.—*John T. Myers.*

SHEEHAN, H. L., and STOREY, G. W. **An improved method of staining leucocyte granules with Sudan black B.** *J. Path. & Bact.*, **59**, 336–7. 1947.

This method appears somewhat more reliable than those previously described, e.g. Discombe (*abs.* Stain Techn., **22**, 121, 1947; compare with Bailiff and Kimbrough, *abs.* Stain Techn., **22**, 120, 1947).

The dry new or old blood film is fixed with formalin vapor by placing it for 5–10 min. in a closed vessel with 4% formalin. It is stained in a solution prepared as follows: Dissolve 0.3 g. Sudan black B in 100 ml. absolute alcohol. Prepare a buffer solution by dissolving 16 g. of phenol crystals in 30 ml. of absolute alcohol; add this phenol solution to 100 ml. of water in which 0.3 g. of Na_2HPO_4-$12H_2O$ is dissolved. Mix 40 ml. of the buffer solution with 60 ml. of the dye solution. The dye must be completely dissolved before mixing. Filter the final solution by suction. The stain solution can be used for several weeks.

The fixed film is immersed in the buffered stain solution in a covered jar for 10–60 min. Slides are washed in absolute alcohol or 70% alcohol for a few min., and are then washed in water. They are counterstained with Giemsa (Gurr's Improved Giemsa R. 66) diluted 1:10 in neutral solution. The blue tint of the plasma and erythrocytes is removed by differentiation 30–60 sec. in 0.2% aqueous KH_2PO_4 solution. The granules stain intense black.—*S. H. Hutner.*

VERDCOURT, B. **An introduction to the study of radulae.** *Microscope*, **6**, 35–9. 1946.

An undetailed review of staining methods for radulae of molluscs is given by the author. Enamel may be removed by a decalcifying agent and the unhardened teeth stained with an acid dye. More popular methods keep the enamel and modify the chitin, usually by oxidation with $KMnO_4$, decolorization with dilute oxalic acid. The radula is then washed with water and stained in dahlia. Chrysoidin, hematoxylin, and chlorazol black E, the latter in either 70% methyl alcohol or saturated aqueous solution, may also be used.

Canada balsam and Euparal are the best mountants. Glycerin jelly is not recommended because of its low refractive index and decalcifying properties.—*C. Randall.*

PLANT MICROTECHNIC

BHADURI, P. N., and BOSE, P. C. **Cyto-genetical investigations in some common cucurbits, with special reference to fragmentation of chromosomes as a physical basis of speciation.** *J. Genetics*, **48**, 237–56. 1947.

Root tips were fixed in Levitsky's fixative, equal parts of 1% chromic acid and 10% formalin or 2 parts 1% chromic acid to 3 parts 10% formalin. Flower buds were pretreated with Carnoy's fluid as modified by Semmens (1 vol. chloroform, 3 vol. alcohol, 1 vol. acetic acid) before fixation in Belling's modification of Navashin's fixative. Paraffin sections stained well with Feulgen light-green or Newton's crystal violet iodine but not with aceto-carmine. Mordanting slides with 1% chromic acid after KI–I treatment improved staining. Several other slight modifications are suggested for specific cucurbits.—*V. Kavanagh.*

VON ROSEN, GOSTA. **A rapid nigrosine method for chromosome counts applicable to growing plant tissues.** *Nature*, **160**, 121–2. 1947.

The author recommends the following method for chromosome counting in roots, leaves, and flower-buds: Fix 24 hr. in 1 part concentrated acetic acid plus 2 parts 95% alcohol. (Roots should be fixed in a cooled fluid). Replace fixing fluid with a cooled mixture of 1 part concentrated HCl plus 2 parts 95% alcohol. (Roots should be treated 8–10 min. For leaves the character of tissue determines concentration and time: *Beta*, 1:2 for 7–8 min.; *Pisum*, 1:4 for 5 min.; *Pyrus* and *Betula*, 1:1 for 10 min.; flower-buds, 1:10 for 5–8 min.). Replace loosening fluid with distilled water and keep in a cool place 15–30 min. Place specimen on object glass and add one drop of staining solution given below. Stain roots 1–2 min.; leaves of *Pisum* 30 sec.; those of *Beta* 40 sec.; *Pyrus* and *Betula* 45–60 sec. Remove excess stain with filter paper.

The staining solution is prepared as follows: dilute 50 ml. concentrated acetic acid with distilled water to 100 ml.; heat to boiling-point; add 4 g. spirit-soluble nigrosin with continuous stirring; boil 3–5 min. to a weak tarry consistency; cool and filter immediately. Keep the solution in a closed glass vessel at room temperature for about 10–14 days before use. For buds only a 3% solution is used.

When this technic is used, chromosomes are stained quite black and the plasma a faint grey.—*C. Randall.*